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14. ABSTRACT Copper speciation in seawater is generally dominated by low concentrations of strong, highly selective Cu chelators. At present, we have no data to account for the high specificity and high binding affinity of these compounds for Cu, nor can we validate hypotheses regarding their sources and sinks. We propose to study the structural and chemical properties of strong Cu chelators produced by a ubiquitous marine cyanobacterium, <i>Synechococcus</i> sp., a plausible source of strong Cu chelators in seawater. We plan to characterize these compounds using high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS), coupled with electrochemical methods currently used to study Cu binding. Dr. James Moffett studies Cu speciation and phytoplankton interactions in seawater and Dr. Nelson Frew is an environmental organic chemist and mass spectroscopist. Such interdisciplinary collaboration is absolutely essential for the proposed work. Results will be used to explain the unique properties of these compounds, which may be important in Cu detoxification. Very few commercially available chelators exhibit the selectivity for Cu displayed by these chelators, suggesting a physiological function. It is possible that these compounds or synthetic analogues may be useful for remediation of Cu contaminated wastewaters, or other processes relevant to the U. S. Navy. Results will also be useful in developing field assays for chelators present in the water column. Such assays are essential in establishing causal links between the accumulation of Cu chelators in seawater and detoxification mechanisms of the biota.					
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# **Characterization of the Structural and Chemical Properties of Copper Chelators in Marine Systems Final Report**

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## **LONG-TERM GOALS**

The long-term goal is to obtain a comprehensive understanding of copper chemistry and bioavailability in seawater, at the molecular level. We are particularly interested in the relationship between water chemistry and biological effects of copper. This information can be used for accurate assessments of the impacts of Cu introduced to harbors by human activities. Such information may also be useful in the development of accurate and economical strategies to detect and remove Cu and other contaminants from waste. Results can be used by dischargers, like the US Navy, and regulators, including the EPA and local agencies, to make informed decisions about managing Cu inputs into harbors and other receiving waters.

## **OBJECTIVES**

The primary objectives of this project relate to naturally occurring Cu binding ligands. Our work, and that of others, shows that these ligands control the variability in Cu bioavailability in many coastal waters. We seek to learn more about the chemical properties of these poorly characterized substances. Current titration methodologies provide information about binding constants and concentrations only. Structural information is necessary to validate hypotheses about sources and sinks (with a view to modeling variability) and to identify compounds with unique properties of relevance to the navy.

## **APPROACH**

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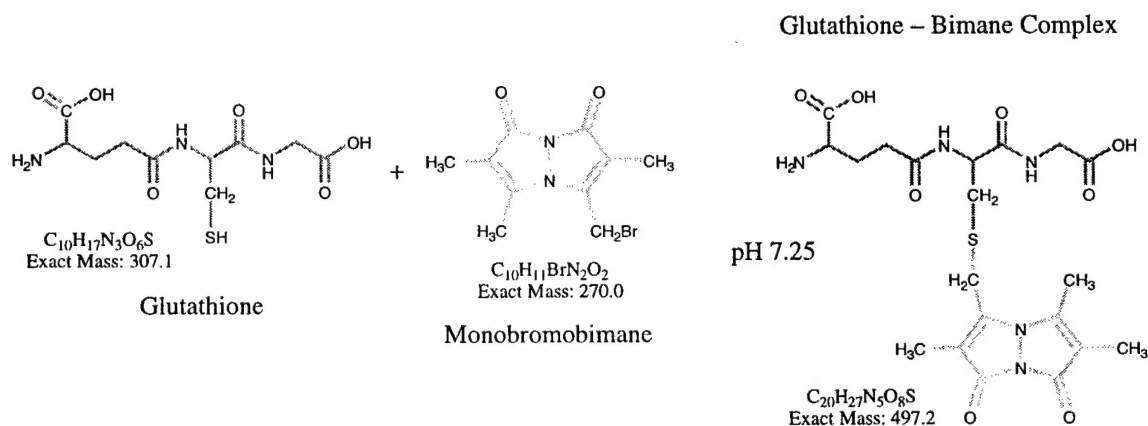
The approach has been to focus on chelators produced by ubiquitous marine phytoplankton and bacteria, rather than chelators actually in the water column, because they can be produced at much higher concentrations. In the past, we have made the case that some organisms may be important sources, such as the marine cyanobacterium *Synechococcus*. Cu stressed *Synechococcus* cultures produce a chelator with binding characteristics similar to the strongest ligands found in the water column. We hypothesized that this ligand is produced as a detoxification mechanism for Cu (Moffett and Brand, 1996).

We have combined measurements of the ligand concentration and thermodynamic properties by cathodic stripping voltammetry, with a several separation schemes to isolate ligands for HPLC analysis.

## WORK COMPLETED

We surveyed a more genetically diverse range of cyanobacteria than in previous years, including halotolerant and freshwater strains, for strong ligand production. We also developed several strategies for precolumn derivatization of metal binding ligands, focusing on sulfhydryl. Ultimately, we settled on derivatization using monobromobimane. We developed protocols to analyze these derivatives by electrospray

## Thiol Derivatization Monobromobimane (mBBr) Reaction



mass spectrometry, and applied our protocols to model ligands and natural samples.

We have been focusing on thiols, in particular an electroactive compound measured in the water column by other workers and attributed to glutathione.

We have studied the reaction kinetics of the unknown compound in comparison to glutathione with oxygen and transition metals as well as the effect of other parameters like pH and light.

The other major task completed was publication of ONR sponsored research. Nine ONR sponsored papers have been published or submitted since last year's report.

## RESULTS

We developed protocols for optimizing production of *Synechococcus* chelators as a function of growth rates, leading to ligand production at levels 100x greater than levels of the strongest ligands in natural waters. However, these levels are still much lower than comparable concentrations of siderophores (Fe binding ligands) produced by bacteria, which have been isolated and characterized. In order to produce more material, we carried out a much wider survey of *Synechococcus* clones, including some freshwater and halotolerant species that are genetically quite distinct from the marine species we had studied before. Previously, all strains we had studied produced strong chelators in response to Cu stress. However, the wider survey revealed that many coastal strains make no chelators at all, evidently having other strategies for detoxification. An interesting generalization has emerged from the study. All of the strains that make the strong ligand contain the light harvesting pigment phycoerythrin (the cultures are pink). All of the strains that don't make the ligand contain phycocyanin (the cultures are green). So far we have no explanation for this observation.

Cathodic stripping voltammetry performed on *Synechococcus* culture media revealed a large peak at  $-0.63$  V (vs. Ag/AgCl). This peak has been observed by others in seawater and eukaryotic cultures and attributed to glutathione. However, many thiol compounds produce a peak at this potential besides glutathione. We investigated the behavior of this peak in the presence of copper and zinc and as a function of pH. We observed a pH dependent shift in potential identical to glutathione. However, addition of Cu or Zn to glutathione results in a disappearance of the peak and appearance of Cu and Zn peaks at different potentials. For the compound in our cultures, the thiol peak disappears but no complex peaks form. This suggests that the compound reacts with Cu and Zn but does not form a complex or that complexes are formed but do not adsorb onto the electrode. We favor the latter interpretation.

We do not know if the peak at  $-0.63$  V contains the strong ligand. It is consistent with work we reported last year on the determination of half wave potentials for Cu complexes in *Synechococcus* cultures that suggested Cu(I) complexes with thiols. This year, we titrated cultures with Cu(I) rather than Cu(II). We reasoned that Cu(II) might simply oxidize thiol ligands (producing Cu(I)). In contrast, addition of Cu(I) would lead to stoichiometric production of a Cu(I) thiol complex. However, results for Cu(I) and Cu(II) were identical despite stringent precautions to exclude oxygen.

Thus we are not sure if the "thiol" peak at  $-0.63$  V is the strong Cu ligand. However, we decided to work on identifying it in order to answer this question conclusively, and because the compound may contribute to the peak at  $-0.63$  V measured by other workers in many marine environments and attributed to glutathione.

## ESMS of derivatized Salt Pond Sample

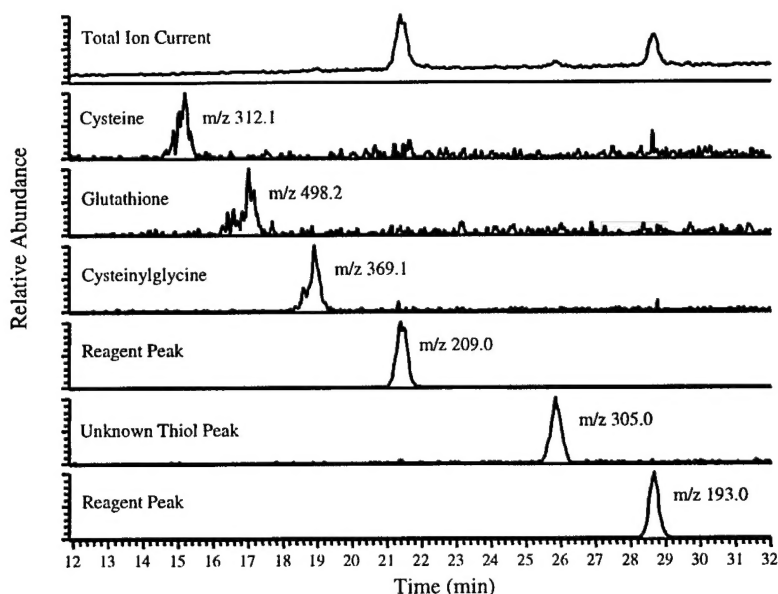
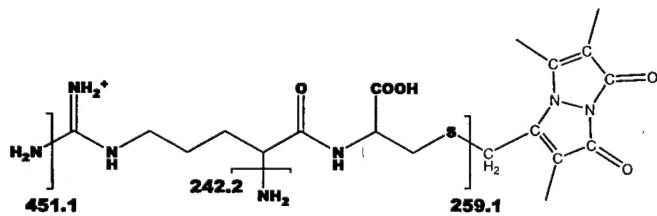
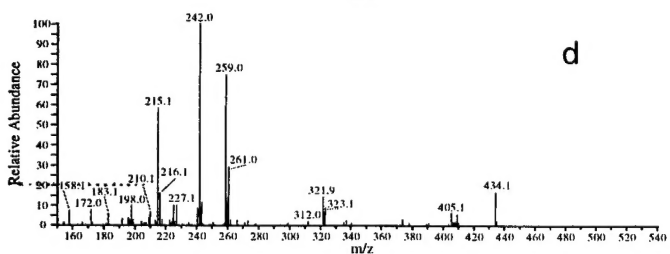
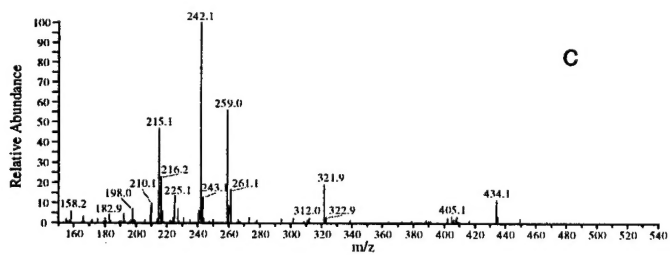
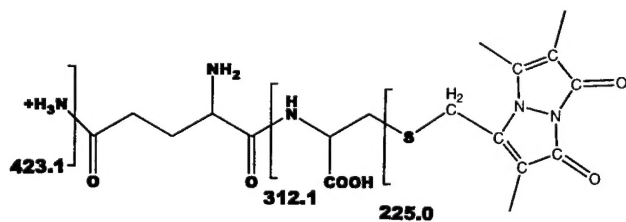
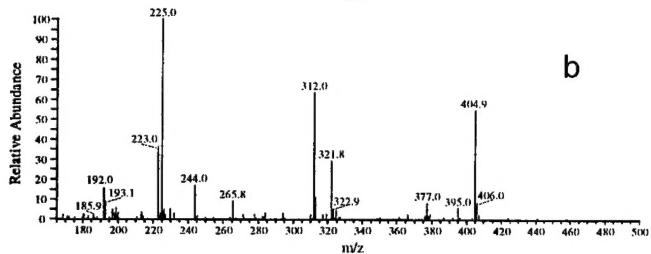
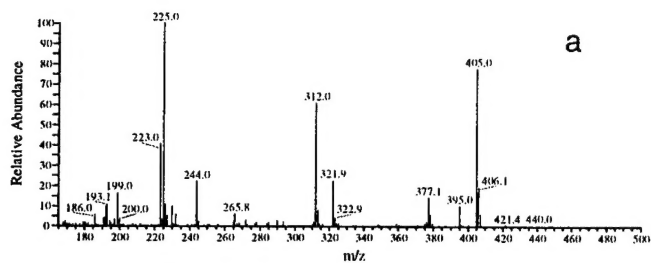


Fig.2. Elution profile of Salt Pond thiol -mBrB derivatives from a reversed -phase C16 Amide Column (Supelco). This chromatogram was produced from a 10  $\mu$ L injection of material concentrated by solid phase extraction (SPE) using C18 Sep Pak cartridges. The concentration factor after SPE was 1000 x.

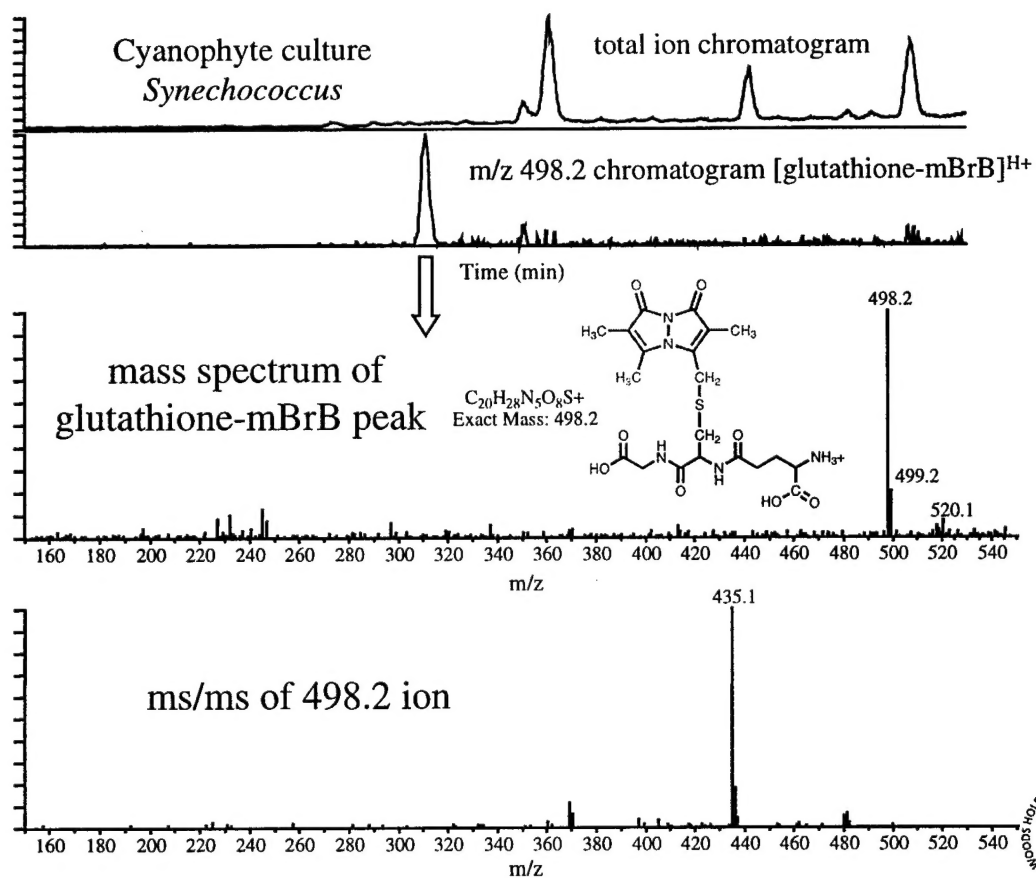
This figure shows a chromatogram of water from Salt Pond, using the mass spec itself as the HPLC detector. Previously, we showed that there were high levels of the “glutathione” voltammetric peak. The results show a thiol in the sample that does not co-elute with any of the “usual suspects” including glutathione. The mass of the adduct is 305, suggesting that this is a low molecular weight thiol of unknown structure. We also were able to detect cysteine, cysteine-glycine and glutathione in the sample, but they were much less abundant than the mystery thiol.

We have also used LC-ESMS to identify thiols produced by the coccolithophore *Emiliania huxleyii*, in collaboration with Beth Ahner (Cornell). Ahner’s group detected two unknown thiols that were produced by *Emiliania* in response to Cu stress. ES-MS of the monobromobimane derivatives revealed two peptides: arginine-cysteine and glycine-cysteine, that were responsible for binding. These sulfides have never been identified before in natural waters, but they may control Cu speciation under some conditions since *Emiliania* is a bloom forming organism.



The figure above depicts MS<sup>3</sup> spectrum of monobromobimane (mBBBr, Molecular Probes) derivatized thiol compounds: unknown 1 (a), synthetic Gln-Cys (b), unknown 2 (c), synthetic Arg-Cys (d). Synthesized compounds are from Cell Essentials, Boston, MA. Structures of derivatized thiols are shown with theorized fragmentation sites. The thiol is shown in bold. The unknown compounds were isolated from *E. huxleyi* (clone CCMP373) cell extracts using pre-column derivatization HPLC<sup>21</sup> coupled to an ESI-ITMS run in positive ion mode (Finnegan Mat LCQ, MS scan  $m/z$ = 100-1000; Spray voltage: 3.52kV; spray current: 4.83mA; capillary voltage: 16.03V; capillary temperature: 134.5oC). The fragmentation patterns for MS and MS<sup>2</sup> of unknown and synthetic compounds are identical, as is the MS<sup>4</sup> of the 312.1 fragment from (a) and (b), which was identified as cysteine by the same method. Original MS  $m/z$  of Gln-Cys/Unk 1 = 440.1, MS<sup>2</sup> = 423.1. Original MS  $m/z$  of Arg-Cys/Unk 2 = 468.2, MS<sup>2</sup> = 451.1.

*Synechococcus* chelators have proven elusive. We have not been able to liberate Cu from these chelators without strong acidification (to pH 2) Mild acidification (to pH 3) is insufficient. When we have done so, glutathione is liberated, which can be readily derivatized (see below). We hypothesize that Cu is complexed in *Synechococcus* cultures as an inert complex with reduced sulfur that only dissociates at low pH, yielding glutathione as a degradation product.





## IMPACT/APPLICATIONS

Successful characterization of this material could lead to new insight into the sources and chemistry of Cu ligands in seawater. Potential applications could arise if we identify a new class of chelators selective for Cu that could be used in remediation.

## TRANSITIONS

None recognized at this time.

## RELATED PROJECTS

Moffett is collaborating with Richard Thompson (U. Maryland) who is supported with 6.2 funds to develop an in situ fiber optic biosensor for Cu. In July 2001 we intercompared his technique with cathodic stripping voltammetry in Eel Pond, MA. We found good agreement. Subsequently, we did more work in the lab and confirmed excellent agreement over a 10000 fold range in free Cu concentration. Moffett also received funds from EPA to develop in situ passive sampling devices to monitor episodic discharge events in harbors. That project was originally "seeded" by a harbor processes grant.

Moffett also works with Brian Palenik(Scripps) on a project to study the relationship between Cu chemistry in seawater and the production of a highly Cu specific cell-surface binding protein by marine diatoms. Palenik has developed an antibody to this protein which may be an excellent in situ indicator of metal stress.

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